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Pathogen-Related Oral Spirochetes from Dental Plaque Are Invasive

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Spirochetes that share pathogen-restricted antigens with *Treponema pallidum* subsp. *pallidum* have been identified in dental plaque and diseased gingival tissues, but it is not known whether these spirochetes possess virulence characteristics. In this study, plaque spirochetes were able to transmigrate a tissue barrier in vitro and were identified on the other side by using monoclonal antibodies specific for pathogen-restricted determinants from *T. pallidum* subsp. *pallidum*. This invasive capability is shared with *T. pallidum* subsp. *pallidum*, but cultured oral and intestinal treponemes did not perforate the tissue barrier. Cocultures indicated that invasive treponemes do not create opportunities for cultivable oral treponemes to cross the barrier. These findings indicate that gingival tissues may be a port of entry for previously unrecognized invasive spirochetes in humans.

Periodontal disease is the most common cause of tooth loss in adults. Because dental plaque, particularly subgingival plaque, is closely associated with diseased periodontal tissues, it is generally assumed that plaque microorganisms or their products are responsible for periodontal disease. Spirochetes are a major microbial component of plaque associated with several forms of periodontal disease (12), including periodontitis that is refractory to nonsurgical treatment (27). Spirochetes have been observed within gingival tissues from diseased sites (3, 4, 9–11, 15), but their identity is unknown.

A positive quantitative correlation has been reported between two serovars of *Treponema denticola* in dental plaque and periodontitis in humans (25, 26). Furthermore, cultivable treponemes, including *T. denticola* (6, 16–19, 24) and *T. vincentii* (14, 19, 24) from the oral cavity and *T. phagedenis* (24) from the intestine, are known to possess degradative enzymes that could contribute to the deterioration of epithelial barriers and, in theory, lead to invasion of tissue. However, at the present time only non-oral pathogenic treponemes are known to be invasive with the ability to disseminate through tissues. *Treponema pallidum* subsp. *pallidum* is able to penetrate cell and tissue barriers both in vivo (1, 8, 13, 23, 28, 29) and in vitro (5, 21, 30). Since monoclonal antibodies (MAbs) previously thought to be specific for known pathogenic treponemes, including *T. pallidum* subspecies and *T. pertenue*, react with pathogen-related oral spirochetes (PROS) in dental plaque (20) and with spirochetes in and around necrotic gingiva (22), it is possible that heretofore undetected oral spirochetes may be invasive. The purpose of this investigation was to determine whether dental plaque collected from patients with chronic adult periodontitis contained invasive spirochetes.

MATERIALS AND METHODS

Subjects and dental plaque. Informed consent was obtained before plaque was collected from adult patients diagnosed by using established criteria as having moderate to severe periodontitis (7). Supragingival dental plaque was removed from tooth surfaces before subgingival plaque was taken from within periodontal pockets. Subgingival plaque was suspended in approximately 1.0 ml of phosphate-buffered saline (pH 7.2) and evaluated for the presence of spirochetes by means of dark-field microscopy. Aliquots of 10 μl of each sample were placed onto glass slides to dry, and the remainder of each plaque suspension was kept at room temperature while each tissue-barrier chamber was assembled. Only plaque samples in which spirochetes were detected with dark-field microscopy were used for invasion studies.

Tissue barrier. Female C3H/He mice were maintained and sacrificed in accordance with institutional guidelines. The abdominal wall was removed under aseptic conditions and assembled between the halves of a dialysis cell to produce a tissue barrier chamber as described previously (21). Dental plaque suspended in phosphate-buffered saline supplemented with 10% normal rabbit serum and 3 μg of rifampin per ml was introduced into the epithelial sides of the chambers. The exit sides of the chambers contained 50% rabbit serum.

Other chambers were challenged with *T. denticola* serovar A (ATCC 35405), *T. denticola* serovar "B" (ATCC 33521), *T. denticola* serovar C (ATCC 33520 and 35404), *T. denticola* serovar D (ST10; the generous gift of R. Smibert, Virginia Polytechnic Institute and State University, Blacksburg), *T. pallidum* subsp. *pallidum* (Nichols strain), *T. phagedenis* (biotype Reiter; the generous gift of J. N. Miller, University of California, Los Angeles), *T. pectinovorum* (ATCC 37768), *T. scoliodontum* (J. N. Miller), *T. socranskii* subsp. *buccale* (ATCC 35534), *T. socranskii* subsp. *socranskii* (ATCC 35536), or *T. vincentii* (ATCC 35580). Cultivable treponemes and *T. pallidum* were prepared as previously described (21), and approximately 10^8 treponemes were introduced into the entry side of each chamber facing the epithelial side of tissue. Each treponeme was tested twice on separate occasions.

Detection of spirochetes. After overnight incubation of chambers at 37°C, wet mounts of entry-side and exit-side chamber fluids were evaluated for motile spirochetes by dark-field microscopy. Aliquots were also dried onto glass slides. Dried specimens, including original plaque suspen-

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TABLE 1. Analyses of treponemes in tissue chambers

Treponeme	Detection of the indicated treponeme in chamber			
	Dark-field microscopy		Immunostaining[a]	
	Entry side	Exit side	Entry side	Exit side
T. denticola				
Serovar A	+	−	+[b]	−
Serovar "B"	+	−	+[c]	−
Serovar C	+	−	+[d]	−
Serovar D	+	−	+[e]	−
T. pectinovorum	+	−	+[f]	−
T. phagedenis	+	−	+[f]	−
T. scoliodontum	+	−	+[f]	−
T. socranskii subsp. *buccale*	+	−	+[f]	−
T. socranskii subsp. *socranskii*	+	−	+[f]	−
T. vincentii	+	−	+[f]	−
T. pallidum	+	−	+[g]	+[g]

[a] Treponemes were dried onto glass slides and then exposed to MAbs in a biotin-streptavidin-alkaline phosphatase immunostain assay (2, 20, 22).
[b] *T. denticola* MAb TDXI,R8B8R8E3 against *T. denticola* ATCC 33520.
[c] *T. denticola* MAb TDII,IAA11 against *T. denticola* ATCC 33521.
[d] *T. denticola* MAb TDIII,IIIBB2 against *T. denticola* ATCC 33520 and 35404.
[e] *T. denticola* MAb TDXIII,R9D9 against *T. denticola* ST10.
[f] Pan-spirochete MAb C2-1, which reacts with a 47-kDa molecule found on all spirochetes tested to date (2, 20).
[g] MAb H9-2, which is against a pathogen-specific epitope on the 37-kDa endoflagellar sheath molecule from *T. pallidum* subsp. *pallidum*.

sions, were examined with immunocytochemistry by using MAb H9-2, which is specific for a pathogen-specific determinant located on the 37-kDa molecule (FlaA) from the endoflagellar sheath of *T. pallidum* subsp. *pallidum*, with MAbs to each of four serovars (A, "B," C, and D) of *T. denticola*, or with MAb C2-1, which is against a pan-spirochete antigen found on all spirochetes examined to date. The derivations and specificities of these MAbs have been described previously (2, 20, 22).

In other experiments, treponemes were radiolabeled as previously described (21, 30), and penetration of the tissue barrier was assessed by dark-field microscopy and by scintillation counting. Each experiment was repeated on separate occasions.

RESULTS

Analysis of control treponemes. Dark-field, scintillation, and immunocytochemical analyses indicated that *T. pallidum* subsp. *pallidum* was able to transverse the tissue barrier but that none of the cultivable treponemes tested was able to pass through the barrier (Tables 1 and 2).

Cocultivation experiments demonstrated that penetration of tissue by *T. pallidum* did not create opportunities for cultivable treponemes to migrate through tissues (Table 3).

Invasive spirochetes in plaque. Not all samples of subgingival plaque from periodontal pockets of patients with periodontitis contained spirochetes. Twenty-eight samples were collected, and nine had no spirochetes detectable with either dark-field microscopy or immunostaining with MAb C2-1. Of the 19 samples with spirochetes, all had spirochetes that reacted with MAb H9-2. Eighteen of 19 spirochete-positive plaque specimens were tested in tissue chambers; mice were not available when one sample was received. Five experiments were lost to contamination. Of the 13 experiments

TABLE 2. Passage of ^{35}S-labeled treponemes through the murine abdominal wall barrier

Treponeme	No. of treponemes (10^7) on the exit side determined by:	
	Dark-field microscopy	Scintillation counting[a]
T. pallidum	4.7	4.7
T. phagedenis	0	0.1[b]
T. scoliodontum	0	0.1
T. denticola ATCC 35404	0	0.1
T. denticola ATCC 35405	0	0.1
T. vincentii	0	0.1
T. socranskii[c]	0	0.1

[a] A total of 5×10^8 treponemes (4.6×10^6 cpm for *T. pallidum*; an average of 5.2×10^6 cpm for other treponemes) were added to the entry side of each chamber for each organism; counts represent averages of data from two chambers.
[b] Background levels of the radioisotope were found with all labeled cultivable spirochetes as well as in chambers incubated with treponeme-free isotope (data not shown).
[c] *T. socranskii* subsp. *socranskii*.

conducted with spirochete-positive and H9-2-positive plaque samples, 12 still had spirochetes detectable with dark-field microscopy on the entry side after incubation and 9 of these also had mobile spirochetes in exit-side fluids after overnight incubation (Table 4). Twelve of 13 spirochete-positive specimens had H9-2-reactive spirochetes in exit-side chambers after culture. Figure 1 is a photomicrograph of an invasive plaque spirochete from an exit-side chamber, stained with H9-2. One subject was tested at two different sites on different occasions, and both samples were H9-2 positive.

Although *T. denticola* isolates were identified with serovar-specific MAbs in starting plaque suspensions, these treponemes were not detected in exit-side fluids with *T. denticola*-specific MAbs (data not shown). No other plaque microorganisms were observed with dark-field microscopy in exit-side chambers.

DISCUSSION

These experiments demonstrate that when dental plaque harvested from sites of periodontitis contains spirochetes, there are spirochetes present that are capable of penetrating and migrating through the mouse abdominal wall, an in vitro model of invasiveness established with *T. pallidum* and the noninvasive intestinal treponeme *T. phagedenis* (21). The entry-side chambers contained a profusion of diverse bacterial forms, including *T. denticola*, but there were no microorganisms other than H9-2-reactive spirochetes in the exit-side chambers. Cultivable treponemes, including *T. denticola*, were not invasive, even when cocultivated with *T. pallidum*.

Since H9-2-reactive oral spirochetes are invasive in vitro, it is likely that they are also capable of invading periodontal tissues through the epithelium of the gingival crevice. There is no reason to believe that penetration of mouse abdominal wall does not represent a process applicable to humans, particularly since the human pathogen *T. pallidum* is also invasive in this model and the nonpathogenic human intestinal spirochete *T. phagedenis* is not invasive. Thus, it is possible that these newly recognized oral spirochetes are the same as those identified in gingival tissues by other investigators using electron microscopy (3, 4, 9–11, 15). This hypothesis is consistent with our observations of PROS in

TABLE 3. Invasiveness of pathogenic treponeme ... mixed treponeme suspensions

Species cocultured with *T. pallidum*	Avg no. of treponemes (10^7) on the exit side determined by:	
	Dark-field microscopy	Scintillation counting[a]
T. vincentii	5.3	0
T. scoliodontum	5.5	0.0002[b]
T. socranskii	5.4	0
T. denticola ATCC 35404	5.3	0.0001
T. denticola ATCC 35405	5.6	0

[a] A total of 5×10^8 unlabeled *T. pallidum* treponemes were mixed with 5×10^8 (3.3×10^7 cpm) of the indicated ^{35}S-labeled oral treponemes. Dark-field counts represent unlabeled *T. pallidum*. Each number represents two experiments.

[b] Background levels of the radioisotope were found with all labeled cultivable spirochetes as well as in chambers incubated with treponeme-free isotope (data not shown).

apparently healthy tissues beyond necrotic gingival lesions (22). However, *T. denticola* was also observed in and around necrotic tissues (22), and it was not possible to determine whether PROS and/or *T. denticola* was opportunistic or invasive. The current investigation helps to resolve this issue by showing that none of the strains of cultivable treponemes studied was able to move across the tissue barrier or appeared to be invasive under these conditions. Of course, bacteria can be pathogenic without being invasive.

The presence of invasive spirochetes in plaque associated with periodontitis may help to explain why periodontitis patients with spirochetes in plaque are resistant to debridement, whereas those patients with few or no spirochetes improve after conventional root planing and other nonsurgical therapy (27). Invasive spirochetes in tissue would be inaccessible to surface treatment, and periodontitis might persist as long as infected tissues remained.

PROS are defined with MAbs against pathogen-restricted determinants found on *T. pallidum* subspecies and *T. pertenue*. We have demonstrated that MAbs to *T. pallidum* subsp. *pallidum* do not react with any cultivable spirochete tested, including those studied in this investigation (2, 20, 22). It is unlikely, therefore, that *Fusobacterium periodonticum*, *T. denticola*, *T. pectinovorum*, *T. phagedenis*, *T. socranskii*, *T. scoliodontum*, or *T. vincentii* was misinterpreted as a PROS. However, there is no evidence that the protein to which MAb H9-2 reacts is directly involved in invasiveness, and it is possible that some pathogenic spirochetes do not react with H9-2.

TABLE 4. Penetration of tissue barrier by plaque spirochetes[a]

Plaque no.	Detection of spirochetes by dark-field microscopy		H9-2 reactivity	
	Entry side	Exit side	Entry side	Exit side
11	+	+	+	+
12, site 1	+	+	+	+
12, site 2	+	−	+	+
13	+	+	+	+
24	+	−	−	−
25	+	+	+	+
31	+	−	+	+
32	+	+	+	+
33	+	+	+	+
34	+	+	+	+
35	+	−	+	+
36	+	+	+	+
41	+	+	+	+

[a] A plaque was not tested unless spirochetes were observable with dark-field microscopy. Overnight cultures of entry-side and exit-side fluids were assessed for spirochetes by dark-field microscopy and by immunocytochemistry with *T. pallidum* MAb H9-2. *T. denticola* was present in entry-side chambers but not in exit-side chambers (data not shown).

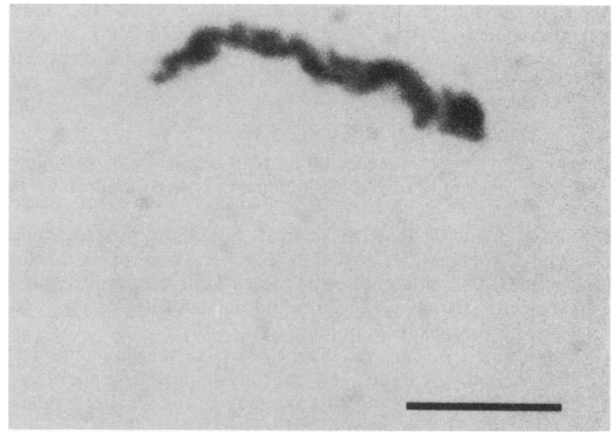

FIG. 1. Photomicrograph of an invasive plaque spirochete from an exit-side chamber. The spirochete was stained with MAb H9-2, which was made against an epitope on the 37-kDa endoflagellar sheath molecule of *T. pallidum*. Bar, 5 μm.

Although PROS share antigens and invasive potential with known pathogenic treponemes, it is unlikely that these subjects had *T. pallidum* or *T. pertenue* in their mouths. All subjects denied a history of treponeme infections, no subject had lesions of oral mucosa, and no subject could be categorized into a group considered at risk for syphilis. In other experiments we have documented evidence of infection by PROS in patients with necrotizing ulcerative gingivitis and periodontitis (20), but patient sera did not react in serologic tests for syphilis (Venereal Disease Research Laboratory, fluorescent treponemal antibody-adsorbed). However, patient sera (but not control sera) did contain immunoglobulin G against 14- and 12-kDa pathogen-restricted antigens. These observations indicate that the PROS is not *T. pallidum* but is phenotypically related to known pathogenic treponemes. More research is needed to define the extent and the nature of this relatedness.

The recognition that the PROS shares certain features with known pathogenic bacteria does not prove that it is the etiologic agent of periodontitis. Moreover, the evidence presented in this report should not be interpreted to indicate that periodontal diseases are a form of syphilis or any other form of venereal or nonvenereal disease caused by *T. pallidum* subspecies and related organisms. Further research is needed to determine whether PROS plays a role in the etiology or pathogenesis of some forms of periodontal disease.

In conclusion, we present evidence for an invasive spirochete associated with periodontitis in humans. This bacterium shares antigens with *T. pallidum* subspecies, but its pathogenic potential has yet to be defined.

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